Quantitative studies of Mn$^{2+}$-promoted specific and non-specific cleavages of a large RNA: Mn$^{2+}$-GAAA ribozymes and the evolution of small ribozymes

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ABSTRACT

Manganese (Mn$^{2+}$) promotes specific cleavage at two major (I and III) and four minor (II, IV, V and VI) sites, in addition to slow non-specific cleavage, in a 659-nucleotide RNA containing the Cr.LSU group I intron. The specific cleavages occurred between G and AAA sequences and thus can be considered Mn$^{2+}$-GAAA ribozymes. We have estimated rates of specific and non-specific cleavages under different conditions. Comparisons of the rates of major-specific and background cleavages gave a maximal specificity of approximately 900 for GAAA cleavage. Both specific and non-specific cleavages showed hyperbolic kinetics and there was no evidence of cooperativity with Mn$^{2+}$ concentration. Interestingly, at site III, Mg$^{2+}$ alone promoted weak, but the same specific cleavage as Mn$^{2+}$. When added with Mn$^{2+}$, Mg$^{2+}$ had a synergistic effect on cleavage at site III, but inhibited cleavage at the other sites. Mn$^{2+}$ cleavage at site III also exhibited lower values of $K_{1/2}$ (Mn$^{2+}$ requirement), pH-dependency and activation energy than did cleavage at the other sites. In contrast, the pH-dependency and activation energy for cleavage at site I was similar to non-specific cleavage. These results increase our understanding of the Mn$^{2+}$-GAAA ribozyme. The implications for evolution of small ribozymes are also discussed.

INTRODUCTION

Divalent metal cations are generally essential for RNA structure, function and catalysis (1–5). These ions can non-specifically surround RNA and shield the negative charges of phosphates. Divalent cations are also located at specific sites in RNA, often where sharp bending of the phosphate backbone or non-canonical helices occur (6–12). Specific metal binding typically involves not only phosphates, but also nucleotide bases of RNA (3) and are important in interlacing distant structural domains (4,5). In addition to the structural role, metals also play central roles in the reactions catalyzed by certain ribozymes (1,5).

The simplest ribozyme-catalyzed reaction is the cleavage of a phosphodiester bond by the adjacent 2′,3′-cyclic phosphate and a 5′-OH, as exemplified by the reactions catalyzed by small naturally occurring ribozymes (e.g. hammerhead, HDV and hairpin ribozymes) (13,14). It has been proposed that Mg$^{2+}$ catalyzes the reaction by polarizing the 2′-OH group, which then initiates nucleophilic attack on the phosphorus, and by stabilizing the incipient transition state (15,16). Divalent metals such as Pb$^{2+}$ (17), Zn$^{2+}$ (18,19), Mn$^{2+}$ (20) and even Mg$^{2+}$ (19,21) can also promote non-biologically relevant site-specific cleavages and/or non-specific degradation of RNA, depending on the conditions. These metal-promoted scission events are similar to the small-ribozyme promoted cleavages, because both leave 2′,3′-cyclic phosphate and 5′-OH termini. Perhaps, nature has utilized the intrinsic reactivity of the 2′-OH and through natural evolution the inefficient and less specific cleavage complexes have lead to the efficient ribozymes we see today. In fact, the in vitro selection of the leadzyme (Pb$^{2+}$-ribozyme) can be regarded as mimicking such an evolutionary process (22).

The smallest, but not well-understood, ribozyme is the Mn$^{2+}$-GAAA ribozyme (23). The minimal structure for activity was shown to be a seven-nucleotide motif, GAAA/UUU (the G is unpaired and the A/Us are Watson–Crick pairs) (24). Mn$^{2+}$ or Cd$^{2+}$ (but not Mg$^{2+}$ or other divalent metals) promoted specific cleavage between G and AAA (23,24). Although Mn$^{2+}$ and Mg$^{2+}$ have similar physicochemical properties and for many natural ribozymes Mn$^{2+}$ can replace Mg$^{2+}$ in supporting their functions (5), this ribozyme prefers Mn$^{2+}$. Mn$^{2+}$ is ‘softer’ and coordinates better with nucleotide bases than Mg$^{2+}$ (3,5), suggesting that there might be sequences (or motifs) which Mn$^{2+}$ preferentially binds and cleaves. There have been comparatively few studies of this ribozyme (25,26), although it has been found in some interesting places (27,28).

Further study of the Mn$^{2+}$-GAAA ribozyme is of interest for several reasons. First, since specificity is an important criterion for enzymes and Mn$^{2+}$ also promotes non-specific RNA cleavage, it is important to know how well GAAA is distinguished from other sequences. Second, the GAAA tetramer is known for forming a stable tetraloop (29), which can also dock with other...
RNA structures as part of tertiary folding (30,31). Also, partially- to highly-conserved GAAA sequences are found in certain small [e.g. hammerhead (32) and hairpin (33)] and large [e.g. group I introns and RNase P (34,35)] ribozymes. Thus, the question arises as to whether these GAAA sequences bind Mn$^{2+}$ or other metals. Third, in the early stages of the proposed ‘RNA World’ (36,37), the complexity of RNA molecules was probably <100 nt (38). The small size of the Mn$^{2+}$-GAAA ribozyme suggests that specific metal-catalyzed RNA cleavage could have been a metabolic tool of the early ribo-organisms (28). Thus, it is of interest to know how a very small ribozyme enhances cleavage rates.

We detected multiple specific cleavages of a large RNA (23S.3 RNA) with Mn$^{2+}$, while studying the in vitro self-splicing of the group I intron in the chloroplast 23S rRNA gene of *Chlamydomonas reinhardtii*, *Cr.LSU* (39,40). Upon mapping the cleavage sites in this 659 nt RNA, we found that they all contained GAAA. Thus, we devised a method to estimate the rates of specific cleavage at several sites and background cleavage under different Mn$^{2+}$/Mg$^{2+}$ concentrations, pH and temperature regimes. These data quantify the specificity of cleavage at GAAA sequences and reveal new properties and variants of the Mn$^{2+}$-GAAA ribozyme.

**MATERIALS AND METHODS**

**In vitro RNA synthesis and purification**

Construction of plasmid pGEM23S.3 was described previously (39). The plasmid contains a 623-bp insert in the pGEM3zf(+) vector (Promega). The insert is comprised of a shortened form of the *Cr.LSU* intron (522 bp, lacking most of the ORF) flanked by exonic sequences. To synthesize the full-length RNA (659 nt), the plasmid was linearized with *Hind*III. For some experiments, it was digested with *Xho*I to produce a 3’-truncated RNA of 481 nt. The transcription reactions (50 µl) were incubated at 37°C and contained 1 mM of each rNTP (A, U, C, G), 3 mM MgCl$_2$, 2 mM spermidine, 10 mM dithiothreitol (DTT), 40 mM Tris–HCl pH 7.5, 2 µg of linearized plasmid DNA and 100 U of T7 RNA polymerase (Boehringer Mannheim). The relatively low MgCl$_2$ concentration was to prevent self-splicing of the RNA during transcription. To prepare internally $^{32}$P-labeled RNA, 10 µCi (1 µl) of $[^{32}]$P-GTP (ICN; specific activity, $\sim$3000 Ci/mmol) were added. After 2 h, the RNA was extracted with phenol/chloroform and precipitated twice with ethanol.

Full-length transcripts were purified by denaturing PAGE (41). The RNA band was located by UV shadowing or by exposing the gel to X-ray film, excised and eluted by incubating the gel slices in 10 mM Tris–HCl pH 7.5, 0.2 M NaCl, 1% (w/v) SDS, 1 mM EDTA at 37°C for several hours. The RNA was precipitated with ethanol after the addition of carrier glycogen (Boehringer Mannheim), re-precipitated twice with sodium acetate and ethanol and dissolved in 10 mM Tris–HCl pH 7.5.

**Mn$^{2+}$-dependent cleavage**

The standard reaction mixture for Mn$^{2+}$-dependent cleavage contained 3 mM MnCl$_2$, (MgCl$_2$-4H$_2$O, BDH), 0.2 M KCl, 50 mM Tris–HCl pH 7.0 (adjusted at 47°C; pK$_a$ of Tris is 7.5 at 47°C) and 1–15 nM $^{32}$P-Labeled RNA (~1000–15 000 c.p.m.) in a final volume of 5 µl. Variations in these conditions are indicated in the legends or text. Immediately prior to the reaction, the RNA (in 10 mM Tris–HCl pH 7.5) was denatured by heating to 95°C for 1.5 min, followed by controlled cooling (~0.5°C/s) to 25°C. Reaction mixtures were set up by aliquotting the renatured RNA into non-siliconized, 0.65-ml microfuge tubes (PGC Scientific), followed by an aliquot of a freshly prepared mixture of the Tris–HCl, MnCl$_2$ and KCl. Pre-mixing MnCl$_2$ with monovalent salt and buffer for long periods of time was avoided since it can accelerate the oxidation and precipitation of Mn$^{2+}$. The standard reactions were incubated at 47°C for 0–100 min, stopped by adding 1.2 vol of gel loading buffer [80% (v/v) formamide, 0.1 M EDTA pH 8.0, 0.1% (w/v) xylene cyanol, 0.1% (w/v) bromophenol blue], heated to 65°C for 3 min, quick-cooled and separated by denaturing PAGE (41).

The following buffers were used to examine the pH dependence: MES, adjusted with NaOH, for pH 5.5, 6.0 and 6.5; HEPES, adjusted with NaOH, for pH 6.5, 7.0 and 7.5; Tris, adjusted with HCl, for pH 7.0, 7.5 and 8.0; and (2-N-cyclohexylamino)ethanesulfonic acid, adjusted with NaOH (CHES) for pH 8.0, 8.5 and 9.0. The final concentration of each buffer was 50 mM. Tris–HCl was used in the temperature-dependence experiments. The pH of the buffers was adjusted at 47°C (or other usage temperature).

**Kinetics of cleavage at multiple sites**

The cleavage reactions, typically ~5000–8000 c.p.m. of internally $^{32}$P-labeled RNA, were separated by denaturing PAGE on long (50 cm) sequencing gels. All of the specific cleavage products and the unreacted 23S.3 RNA were quantified with a Phosphorimager ( Molecular Dynamics) and ImageQuant software ( Molecular Dynamics). A range of uncleaved RNA amounts (10–15 000 c.p.m.) were analyzed in parallel and used to establish a standard curve for obtaining the c.p.m. of the RNA bands.

Initial rates of cleavage at specific sites were used to obtain the $k_{obs}$. In general, data from cleavage of less than the first 20% of the input RNA was used, and under most conditions >80% of the RNA cleaved during this time was at specific sites. However, at the upper extremes of pH, Mn$^{2+}$ concentration and temperature, non-specific degradation became significant (up to 60% of the cleaved RNA). Thus, the measurements of specific cleavage products (c.p.m.) were corrected (in all conditions) for the contribution of non-specific cleavage. The rate of non-specific cleavage of 23S.3 RNA was also of interest for comparative purposes and was estimated as follows. It was assumed that the rate of non-specific cleavage was approximately equal for all phosphodiester in 23S.3 and that at the early time points degradation of the intact RNA was principally by single, non-specific cleavage events. Thus, the following equation applied:

$$\ln((\Sigma R)/R_0) = -658 k_{obs} t$$  

where $R_0$ is the initial amount of radioactive 23S.3 RNA, $\Sigma R$ is the sum of the specific cleavage products and the remaining intact RNA (i.e. the RNA that was not non-specifically cleaved), 658 is the number of phosphodiester in 23S.3 and $t$ is time. The ratio $(\Sigma R)/R_0$ is related to the fraction of 23S.3 RNA non-specifically cleaved by the following:

$$f_{ns} = 1 - (\Sigma R)/R_0$$
non-specific cleavage of the RNA. Thus, the slopes of semi-log plots of $(2R)/R_0$ with time provided the $k_{obs}$ for non-specific cleavage.

The direct measurements of specific cleavage products provided an underestimate of the real site-specific cleavage rate because the specific cleavage products were also subjected to non-specific cleavage during the reaction. Thus, to obtain the $k_{obs}$ for cleavage at a specific site, the term $S$, which gives the corrected amount of a specific cleavage product, was used:

$$S = \frac{R_f}{m_f \cdot (R_0 / m_P)}$$

where $R_0$ is the amount of the specific cleavage fragment, $m_f$ is the number of Gs in 23S.3 RNA, $m_P$ is the number of Gs in the cleavage fragment and $f_{m_P}$ is the fraction of 23S.3 non-specifically cleaved (which is described by equation 2). The components of ‘$S$’ can be understood as follows: the numerator, $[(R_f / m_f) / (R_0 / m_P)]$, is the observed fraction of 23S.3 RNA specifically cleaved at the site in question, whereas the denominator, $[1 – f_{m_P} (m_f / m_P)]$, corrects for the effect of non-specific cleavage on the specific cleavage product. The latter component is based on equation 2 and takes into account the fact that the rate of disappearance of the specific cleavage product is length-dependent [hence, the $(m_f / m_P)$ term]. Note that $S$ is independent of reaction volume and has no units. At the initial time points, $dS/dt = k_{obs}$. Thus, a reasonably good estimate of $k_{obs}$ was obtained from the slope of $S$ versus initial time plots. Specific cleavage at different sites in 23S.3 RNA, which were estimated simultaneously, were treated as independent events. The rate constants determined by this method were not significantly affected by RNA concentrations over the range employed (1–50 nM). The derivation and validity of these equations was discussed in more detail elsewhere (42). Kaleidograph software (Synergy Software) was used for curve fitting.

**Primer-extension analysis**

Primer-extension analysis of RNA was performed as described by Christopher and Hallick (43), except for the following modifications. The reactions contained 0.6 µCi of [α-32P]dCTP (ICN; specific activity, 3000 Ci/mmol), 100–150 ng of RNA and 10 ng of non-radioactive oligonucleotide primer. The following oligonucleotides were used: #8, (5′-dTATATTGT-TATTGATAAGTATG-3′), which anneals to nt 176–196 of the intron in 23S.3; #37, (5′-dCAGGAGTCCGCGGTATA-3′), which anneals to nt 406–422 of the intron in 23S.3; and #95, (5′-dTGCCTGCAGGTCGACTCTAAGA-3′), which anneals to the 3′ exon, 16–36 nt from the 3′ splice site. The extension reactions were performed for 30 min at either 42°C (oligo #8), 47°C (oligo #37) or 50°C (oligo #95). Sequence ladders were generated by adding a deoxynucleotide (final concentration, 10 µM) to reactions with untreated RNA. The reactions were stopped by adding 7.5 µl of gel loading buffer (see above), heating to 95°C and denaturing PAGE.

**Enzymatic sequencing of end-labeled RNA**

Non-radioactive 23S.3 RNA was prepared, cleaved with Mn2+ under standard conditions, and then end-labeled with T4 polynucleotide kinase and [γ-32P]ATP (41). The RNAs were purified by denaturing PAGE as described above, cleaved with base-specific RNases (44) and the products separated on 20% denaturing gels. The wet gels were transferred to used X-ray film, wrapped in Saran wrap and exposed to X-ray film (BioMax MS, Kodak) at −70°C with DuPont Cronex intensifying screens.

**RESULTS**

**Mn2+-dependent cleavage of 23S.3 RNA**

Figure 1A is a linear diagram of 23S.3 RNA, showing the positions of the specific cleavage sites (identified below). Figure 1B shows the cleavage pattern obtained when 23S.3 RNA was incubated with Mn2+ under standard cleavage conditions (3 mM MnCl2, 0.2 M KCl, 50 mM Tris–HCl pH 7.0, 47°C) for 0–80 min. There were four major fragments and a number of minor ones. Detailed mapping revealed six cleavage sites, I–VI (see below). The major cleavage sites are I and III, and the minor ones, sites II, IV, V and VI (Fig. 1A). Accordingly, the RNAs derived from a single cleavage of the precursor are annotated by site and whether they contain the 5′ or 3′ end of the RNA [e.g. I(5′), I(3′), etc.]. RNAs produced by cleavage of 23S.3 at two sites are denoted with a hyphen; for example, the 228-nt RNA denoted I-III, is produced by cleavage at both major sites (I and III). All of the products of single cleavages, with the exception of the 558-nt site VI(5′) fragment (which was too weak to be identified with confidence) and most of the expected products of double-cleavage (one major-one minor) could be found. Finally, the RNAs are also named in order of descending size (f1, f2, etc.).

The parameters of the standard cleavage reaction, i.e. MnCl2, monovalent salt, pH and temperature, were chosen to obtain the greatest amount of specific cleavage in a 40-min reaction with minimal requirements. The effects of Mn2+ concentration, pH and temperature on the specific cleavage of 23S.3 RNA are presented below. Monovalent salts (KCl or NaCl), up to at least 0.5 M, increased the proportion of specific cleavage by reducing background cleavage (data not shown).

**Localization of the cleavage sites**

To facilitate mapping of the cleavage sites, preliminary experiments were performed by cleaving 5′-end-labeled 23S.3 RNA with Mn2+ or by post-cleavage labeling of non-radioactive cleavage products with [γ-32P]ATP and polynucleotide kinase. These results indicated that the major cleavage sites were ~150 and ~375 nt, respectively, from the 5′ end of 23S.3 and that minor cleavage sites were located between the two major sites and between the second major site and the 3′ end of 23S.3 RNA (data not shown).

This information was used to design oligonucleotides for primer extension analysis (Fig. 2A). Figure 2B shows the results with oligonucleotide #8. A major product was obtained specifically with Mn2+-treated RNA (Fig. 2B, lanes 6 and 7), which indicated that cleavage occurred between G146 and A150. To precisely locate the other major cleavage site (and minor sites), oligonucleotide #37 (Fig. 2A) was used. Figure 2C, lanes 6 and 7, show that a doublet of major bands and two minor products were specifically obtained with Mn2+-treated RNA. The positions of the doublet suggested that there were adjacent cleavage sites between U375 and G377 and between G377 and A378, respectively (however, see below). The positions of the two minor cDNAs indicated that cleavages also occurred between G344 and A345 and between G385 and A386.

Primer extension was also performed on the f7 cleavage product of 23S.3 RNA. The positions of the doublet suggested that there were adjacent cleavage sites between G344 and A345 and between G385 and A386.
purified by denaturing PAGE. The result (Fig. 2C, lane 5) was the same as that obtained with total, cleaved 23S.3 RNA (Fig. 2C, lanes 6 and 7), confirming that f7 is a product of the second major cleavage. The absence of the two minor cDNA products from the extension of f7 is consistent with their resulting from cleavages of 23S.3 RNA at other sites. Oligonucleotide #95 was used to map cleavage sites in the 3′ portion.
of 23S.3 RNA. Figure 2D and E show that Mn²⁺-dependent cleavage occurred between G⁴⁹² and A⁴⁹³ and between G⁵⁵⁸ and A⁵⁵⁹, respectively. The signal at the latter site was weak, but reproducible nonetheless.

Direct RNA sequencing was used to independently confirm positions of the Mn²⁺ cleavage sites. The size (∼230 nt) of the f₁₀ RNA (Fig. 1) suggested that it was derived by double-cleavage of 23S.3 RNA at the two major sites. This RNA was isolated after Mn²⁺ cleavage and 5′-end-labeling and subjected to enzymatic sequencing. The results, which are shown in Figure 2F, indicate that the first 5 nt of f₁₀ are 5′-AAACC-3′. This result is consistent with the primer extension analysis and confirms the position of the first major cleavage site. The primer-extension data also suggested that the second major cleavage site consisted of two adjacent cleavages, one preceding and one following G₃⁷⁷, and that there was approximately equal cleavage at these two linkages. This was examined further by subjecting end-labeled f₇ RNA to enzymatic sequencing. The results, which are shown in Figure 2G, indicate that this RNA begins with A and there was no evidence for an RNA with a 5′-terminal G. These data indicate that the cleavage at the second major site is not a doublet but is similar to the others in occurring primarily between G and A. The doublet of bands produced during primer extension can be attributed to reverse transcriptase adding a non-templated nucleotide to the end of the cDNA (45). It is not known why this occurred more efficiently with this substrate than with the other major extension products. Finally, cleavage after G⁴⁹² (Fig. 2D) was examined by direct sequencing of end-labeled f₁₃ RNA (Fig. 1B) and the results were consistent with the primer extension analysis (data not shown).

Table 1 shows the sequences at the six cleavage sites; GAAA is the consensus sequence and cleavage occurred between G and AAA. It should also be noted that these are the only GAAA sequences in 23S.3 RNA. Thus, specific cleavage of 23S.3 RNA by Mn²⁺ occurred primarily and at all GAAA sequences.

<table>
<thead>
<tr>
<th>Site</th>
<th>Sequence</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>GGU₁₄⁹↓AAA⁺CU</td>
<td>J₄/5</td>
</tr>
<tr>
<td>II</td>
<td>UCU₃₄⁴↓AAA⁺CG</td>
<td>P₆b extension</td>
</tr>
<tr>
<td>III²</td>
<td>AGU₃₇⁷↓AAA⁺AAA</td>
<td>P₆b extension</td>
</tr>
<tr>
<td>IV</td>
<td>AAA₁₅⁰↓AAA⁺UCG</td>
<td>P₆b</td>
</tr>
<tr>
<td>V</td>
<td>UGG₄₉₂↓AAA⁺GUA</td>
<td>P₇.2</td>
</tr>
<tr>
<td>VI</td>
<td>UAC₃₉₅↓AAA⁺GUA</td>
<td>L₉</td>
</tr>
</tbody>
</table>

Quantitative characterization of cleavage events

The 23S.3 RNA decreased with first order kinetics (t₁/₂ = 13 ± 1 min under standard conditions; Fig. 1B) during Mn²⁺-cleavage and was apparently homogeneous. Thus, we developed an approach to estimate the observed rate constants (kₐₒbs) for cleavage at multiple sites simultaneously using data collected at early time points. Figure 3A shows the early accumulation of products of specific cleavage at sites I, III, IV and V;
cleavage rates at sites II and VI were too low to be reliably estimated. All four lines are close to linear over this period. Under most conditions, >80% of the cleavage at early time points was due to site-specific cleavage; however, at the upper extremes of Mn\(^{2+}\) concentration, pH and temperature, non-specific cleavage became significant. Thus, the measured values of specific cleavage products were corrected for the effect of non-specific degradation (see Materials and Methods). This practice significantly expanded the range of each parameter that was analyzed. It also provided non-specific cleavage rates for comparisons.

Figure 3B shows the rate constants (\(k_{\text{obs}}\)) for cleavage at sites I, III, IV and V as a function of Mn\(^{2+}\) concentration. Hyperbolic curves were obtained and there was no evidence of cooperativity. Cleavage rates at the major sites (I and III) plateaued at ~3 mM Mn\(^{3+}\). The slopes of log–log plots of the data before saturation gave values close to one for cleavage at the specific sites and for non-specific cleavage.

The kinetic parameters, \(k_{\text{cat}}\) and \(K_{\text{m}}\), for cleavage at each site are given in Table 2. Site I had the highest \(k_{\text{cat}}\) and site IV the lowest, but with only a 6-fold difference between them. The lowest \(K_{\text{m}}\) (0.3 mM) belonged to site III, suggesting a greater affinity for the metal at this site compared to the others. Thus, although the \(k_{\text{cat}}\) for site III is only 1.8-fold greater than for site V, the considerably greater cleavage at site III under standard conditions (Fig. 1) can be explained by the fact that the Mn\(^{3+}\) concentration was nearly saturating for site III, but not for site V.

Table 2. Kinetic parameters for Mn\(^{3+}\)-dependent cleavage of 23S.3 RNA at sites I, III, IV and V

<table>
<thead>
<tr>
<th></th>
<th>I</th>
<th>III</th>
<th>IV</th>
<th>V</th>
<th>Non-specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>(k_{\text{cat}}) (×10^3 min(^{-1}))</td>
<td>18</td>
<td>9</td>
<td>3</td>
<td>5</td>
<td>0.14</td>
</tr>
<tr>
<td>(K_{\text{m}}) (mM)</td>
<td>1</td>
<td>0.3</td>
<td>3</td>
<td>3</td>
<td>7</td>
</tr>
</tbody>
</table>

The values are means of four determinations that varied no more than 20%. \(k_{\text{obs}}\) is the Mn\(^{3+}\) concentration at which \(k_{\text{obs}} = \frac{1}{2} k_{\text{cat}}\).

Figure 3C shows how cleavage specificity varied as a function of Mn\(^{3+}\) concentration for the major cleavage sites. Specificity is defined as the ratio of \(k_{\text{obs}}\) for specific cleavage over the \(k_{\text{obs}}\) for non-specific cleavage. The graphs show that specificity peaked at low (0.3–0.5 mM) Mn\(^{3+}\) concentrations and the highest specificity was for site III (0.3 mM Mn\(^{3+}\)), where the cleavage rate was ~900-fold greater than non-specific cleavage.

Figure 4A shows the effect of varying pH on Mn\(^{3+}\)-dependent cleavage. Cleavage rates at specific sites (and non-specific cleavage) increased linearly from pH 5.5 to 8.5, except for site I, which plateaued at pH 8 (Fig. 4A). The slopes of the pH–\(k_{\text{obs}}\) plots in the linear portion of the curves were 1 for site I and 0.8 for sites IV, V and non-specific cleavage. These results indicate that, for cleavage at these sites, a single deprotonation event is the major rate-limiting step (46). Interestingly, the slope of the pH–\(k_{\text{obs}}\) plot for site III was only ~0.5. One possible explanation of such a low pH–rate dependency is that a protonation step (which is required for the departure of the 5′-OH leaving group) is not much faster than the deprotonation step. In this regard, we note the evidence of Zhou et al. (47) that departure of the 5′ leaving group was limiting for a hammerhead ribozyme. The rate plateau at high pH could result from some precipitation/oxidation of the Mn\(^{3+}\) (48) or a change in the rate-limiting step.

The effect of temperature on Mn\(^{3+}\)-dependent cleavages are shown in Figure 4B. The Arrhenius plots of the effect of temperature on Mn\(^{3+}\)-dependent cleavage at sites I, III, IV and V (compared to nsp cleavage). 23S.3 RNA was incubated under standard cleavage conditions except for varying the temperature from 25 to 70°C. The lines were drawn by performing regression analysis on the data obtained from 30 to 65°C.
similar to non-specific (29.6 kcal/mol) and, therefore, most of the rate enhancement is attributable to an increase in the frequency factor, which can be described as the frequency of collisions with the correct orientation. It is also interesting to note that the activation energy for site III cleavage is substantially (~2-fold) lower than for the other sites, including site I.

Effects of Mg\(^{2+}\) on Mn\(^{2+}\)-dependent cleavage

Figure 5A shows the effects of increasing Mg\(^{2+}\) concentration on Mn\(^{2+}\)-dependent cleavage of internally-labeled 23S.3 RNA. The cleavages at sites I, IV and V were inhibited by Mg\(^{2+}\) (>1 mM), whereas cleavage at site III was first stimulated at low Mg\(^{2+}\) (<25 mM) and then gradually decreased at higher Mg\(^{2+}\) concentrations. Interestingly, as Figure 5A and B (lanes 11, 12 and lane 10, respectively) shows, Mg\(^{2+}\) alone promoted cleavage at site III and at the same phosphodiester as Mn\(^{2+}\).

Figure 5A, lane 5, also shows that as the Mg\(^{2+}\) concentration was raised to 12.5 mM, the group I ribozyme activity of 23S.3 RNA was induced, as indicated by the appearance of characteristic products [linear and cyclized intron and 5' exon molecules (39)]. A ligated exon molecule was not formed to any significant extent because there was no guanosine in the reaction. This result indicates that Mg\(^{2+}\) promoted 23S.3 RNA to fold into a native conformation.

Rates of Mg\(^{2+}\)-dependent cleavage at site III were quantified by using a XhoI-truncated 23S.3 RNA, which terminates in P7.2 and has no group I ribozyme activity. The kinetic parameters of Mn\(^{2+}\)-dependent cleavage at sites I and III were essentially unchanged in this shortened RNA (data not shown). The \(k_{cat}\) and \(K_{m}\) of Mg\(^{2+}\)-dependent cleavage (at site III) were ~10-fold lower and ~16-fold higher, respectively, than those of Mn\(^{2+}\)-promoted cleavage at site III (data not shown). These data indicate that the binding and catalysis by Mg\(^{2+}\) at site III are much weaker than Mn\(^{2+}\).

DISCUSSION

Extraordinary specificity at GAAA sites

The primary sequence of this 659-nt RNA contains 229 different tetramers (out of a possible 256), including 11 single-point-variants of GAAA (out of a possible 12), yet none were efficiently cleaved by Mn\(^{2+}\). In addition, cleavage of several other RNAs (650–1650 nt) with Mn\(^{2+}\) gave products consistent with the distribution of GAAA sequences (unpublished data). Indeed, we have shown that Mn\(^{2+}\) cleaved 23S.3 RNA at the major-site GAAA \(\sim 900\)-fold faster than other sequences.

Mn\(^{2+}\)-binding at cleavage sites

The binding of Mn\(^{2+}\) to these sites is probably confined to the tetranucleotide and does not involve a large structural rearrangement. This is suggested by the lack of cooperativity in the rate–concentration curves. Hyperbolic kinetics were also observed in the cleavage of a pentanucleotide (GAAACp) by poly(U) and Mn\(^{2+}\) (25). It is unlikely that the 0.2 M KCl in the cleavage reactions suppressed a cooperative effect, because there was evidence of such an effect at site III by Mg\(^{2+}\), although not by Mn\(^{2+}\). It should be noted that the low Hill coefficient (\(n = 1\)–1.0), which we and others (25) have observed, does not necessary preclude models with multiple Mn\(^{2+}\) binding to GAAA (3,24).

The numbers of RNA-bound metals estimated by activity

Figure 5. The effects of Mg\(^{2+}\) on Mn\(^{2+}\)-dependent cleavage at specific sites. Mg\(^{2+}\) induces cleavage at site III. (A) Analysis with \(^{32}\)P-labeled RNA. Internally-labeled 23S.3 RNA was incubated under standard cleavage conditions plus the indicated concentrations of Mg\(^{2+}\) in mM (lanes 2–10). The RNA was also incubated with Mg\(^{2+}\) as sole divalent cation (lanes 11 and 12) and without divalent cations (lane 1). The major Mn\(^{2+}\)-cleavage products are labeled to the left as in Figure 1. The group I ribozyme-related reaction products are: C, circularized intron; E–I, 5' exon–intron; I, linear intron; 5'E, 5' exon. The RNA species (*) migrating just above the I-III product is due to Mn\(^{2+}\) cleavage of the linear intron at site III. The sizes of the RNAs are given (in nt) between lanes 10 and 11. (B) Primer extension analysis of non-radioactive RNA. Unlabeled 23S.3 RNA was incubated with Mn\(^{2+}\) and/or Mg\(^{2+}\) at the indicated concentrations (the other conditions were standard) and then primer extension was performed with oligonucleotide #37 (Fig. 2) and [\(\gamma\)-\(^{32}\)P]CTP. The extension products derived from cleavage of the RNA at sites III and IV are indicated to the left. The sequence ladder (lanes 1–4) was generated with the same primer on untreated 23S.3 RNA.
Cleavage efficiency and GAAA conformation

In an attempt to explain the differences of cleavage efficiencies at each site, the secondary structures of these sites were inferred from the established secondary structures for group I introns (34). As Figure 6 shows, sites I, IV, V and VI lie in the conserved J4/5, P6b, P7.2 and L9 regions (Fig. 6A), respectively, whereas sites II and III are in a non-conserved extension of P6b (Fig. 6B). The two major cleavage sites, I and III, are located within an internal loop and a four-way junction, respectively, structures which often bring about turns and twists of RNA helices (1,5) and could possibly mediate strong metal binding. This may account for the high cleavage efficiencies at these sites. Interestingly, the AAA trinucleotide at these sites apparently does not pair with UUU as in sites IV and V and in the original ribozyme (23,24,27). Presumably, the GAAA at these sites can adopt the active conformation without pairing with UUU. The weaker cleavages at sites IV and V are consistent with the notion that regular RNA helices usually are not ideal locations for specific metal binding (4). The weakest cleavage (at site VI) is in a stable tetraloop (30,31), which may bind divalent metals only weakly (54). The extremely poor cleavage at this site may be due to the absence of available sites for metal coordination; the N-7 atoms of A2 and A3, which appear to be important for the GAAA/UUU ribozyme (25), should be involved in interactions within the tetraloop (30,55).

It is not clear from the secondary structure of site II why cleavage at this site was weak. However, this site, which is located in a terminal loop, may be involved in forming a pseudoknot with 318GGUUU321 (Fig. 6B). It is also possible that some of the proposed secondary structure in the P6b extension (Fig. 6B) is incorrect, since it is based only on a thermodynamic prediction (unlike the conserved core in Fig. 6A).

While the secondary structures of the cleavage sites can be approximately correlated with cleavage efficiencies, the tertiary structure at these sites will determine the actual metal binding and cleavage. Thus, it would be informative to know how Mn2+ affects tertiary folding. Unfortunately, Fenton reagents, which have been applied to study the tertiary folding of Tl.LSU (50) and phage (56) group I ribozymes, cannot be used in the presence of Mn2+. Alternatively, we examined the structures of RNA where the conformation of GAAA is known. The crystal structure of the P4–P6 domain of the...
To find that the activation energy for specific cleavage at site I (and sites IV and V) was similar to that of background cleavage. However, reactions can also be accelerated by increasing the concentration of reactants and by properly orienting the reactants at the catalytic sites. It appears that by forming a metal-binding pocket at site I, the local concentration of Mn$^{2+}$, and the probability of the right orientation, was raised. The weaker cleavages at sites IV and V may reflect the frequencies of conformational transitions from a regular helix to a non-canonical helix (as adopted by site I).

**Metal binding in group I introns**

In the present study, we have identified two major Mn$^{2+}$-binding sites in 23S.3 RNA and, interestingly, one is located in the conserved J4/5 region where a GAAA sequence is found in many group I introns (34). In the crystal structure of the P4-P6 domain of *Tl.LSU*, a Mg$^{2+}$ was located near the phosphate of the G in the J4/5 GAAA sequence (12), and in 23S.3 RNA, Mn$^{2+}$ inhibited Mn$^{2+}$-promoted cleavage at this site. Taken together, the data suggest that J4/5 (or part of J4/5) is in (or near) a divalent metal binding site in many group I introns. When the J4/5 GAAA of 23S.3 RNA was mutated to GACA, specific cleavage by Mn$^{2+}$ was abolished and the Mg$^{2+}$-requirement for *in vitro* self-splicing was raised dramatically (T.-C. Kuo, S. Holloway and D. Herrin, unpublished data; 63). Thus, the posited role of metal binding at J4/5 is structural, rather than catalytic. It is not known, however, if the conformation of J4/5 GAAA (or the whole 23S.3 RNA) is the same in Mn$^{2+}$ as in Mg$^{2+}$. This question arises because Mn$^{2+}$ alone does not support the self-splicing of *Cr.LSU*, *Cr.psbA2* or *Cr.psbA3* (42) and at high concentrations Mn$^{2+}$ inhibits the self-splicing of *Tl.LSU* (64), *Cr.psbA1* and *Cr.psbA4* introns (42). Also, whereas Mn$^{2+}$ promoted specific cleavage in the conserved J8/7 region of a T4 phage group I intron (65), it did not in 23S.3. Thus, not all self-splicing group I introns fold the same in the presence of Mn$^{2+}$. More structural studies are required to address the effects of Mn$^{2+}$ on folding of 23S.3 RNA.

**Evolution of the RNA World**

The specificity of Mn$^{2+}$-promoted GAAA cleavage suggests that in the ancient RNA World, metal-dependent site-specific cleavage could have been performed by ribo-organisms with genome sizes of only \(\sim 100\) nt (38). The likelihood of forming a simple metal-binding pocket (e.g. cleavage site I in this study) must be relatively high, in part because other divalent metals besides Mn$^{2+}$ are known to promote site-specific cleavages in RNA of different sizes [e.g. 30-nt leadzyme (22), 76-nt tRNA (17,20), 377-nt RNase P (19) and rRNAs, 16S and 23S, (66)]. However, in environments replete with metals that can also promote non-specific RNA degradation, the population and complexity (i.e. genome size) of ribo-organisms would increase only if they were equipped with highly specific ribozymes. Therefore, under the appropriate selection pressure, more complex ribozymes (e.g. cleavage site III and leadzymes) would have evolved. The hairpin and hammerhead ribozymes could be regarded as two of nature’s final choices. The cleavage reactions catalyzed by these ribozymes have low activation energies [e.g. 13–22 and 18–19 kcal/mol for hammerhead and hairpin ribozymes, respectively, (67–70)], relatively high \(k_{\text{cat}}\) values [1.5 and 2.1 min\(^{-1}\) for hammerhead and hairpin ribozymes, respectively (71,72)] and very low pH-dependency.
[e.g. hairpin ribozyme (70)]. More strikingly, they can use monovalent salts to promote catalysis (73). Finally, it should be noted that the fact that GAAA sequences do not have to be cleaved increased the potential usefulness of this motif in the ancient and modern RNA Worlds.

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REFERENCES